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Award Number: W81XWH-07-1-0218

TITLE: Non-Invasive Nanodiagnostics of Cancer (NINOC)

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REPORT DATE: June 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

     x Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* 01-04--2009	2. REPORT TYPE Annual	3. DATES COVERED *(From - To)* 01 Apr 2008 – 31 Mar 2009

4. TITLE AND SUBTITLE Non-Invasive Nanodiagnostics of Cancer	5a. CONTRACT NUMBER
	5b. GRANT NUMBER W81XWH-07-1-0218
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Alexander Kabanov Email: ksutton@unmc.edu	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Nebraska Medical Center Omaha, NE 68198-7835	8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) US Army Medical Research and Materiel Command Fort Derrick, MD 21702-5012	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This project seeks to develop noninvasive diagnostics to detect cancer in its earliest, most easily treatable, pre-symptomatic stage. Innovative imaging nanomaterials and delivery technologies are employed to target, label, and detect the cancer cells within the body at sites which are normally inaccessible to conventional diagnostic methods. The hydrophilic polymer nanogels of core-shell emission computed tomography, computer tomography, magnetic resonance, or luminescence detection. The surface of the nanogels is modified with genetically engineered antibody fragments to target the surface of cancer cells and provide site-specific delivery of the nanogels to tumors in the body.

15. SUBJECT TERMS
Noninvasive diagnostics to detect cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Keith Sutton
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	17	19b. TELEPHONE NUMBER *(include area code)* 402-559-3455

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

To improve the detection and treatment of cancer, tumor-specific targeting has been proposed using a variety of targeting moieties such as folic acid, transferrin, RGD-peptides, antibodies or their fragments. A typical targeting method is to use antibodies to target cancer cellular markers. Maximal tumor targeting with minimal background or minimal exposure of normal organs is the goal for the clinical application of monoclonal antibodies (MAbs) for cancer diagnosis and therapy. MAbs and their fragments are of particular interest due to their high specificity for their epitopes, as well as wide variety of possible target structures. In addition, successful development of genetically engineered humanized antibody fragments reduces the problems with immune responses against mouse antibodies [1].

Tumor-associated mucines have been implicated in the pathogenesis of many cancers. Tumor-associated glycoprotein (TAG-72) is a panadeno-carcinoma antigen, which is expressed by majority of primary and metastatic human adenocarcinomas. It has been demonstrated that TAG-72 is expressed on about 85% of human adenocarcinomas such as colon, breast, pancreatic, ovarian, prostate, non-small cell lung, and gastric cancers and is not expressed in normal adult tissues except secretary endometrial tissues [2].

The murine MAb CC49 recognizes the sialyl-Tn and sialyl-T epitopes [3], which are disaccharide carbohydrate antigens present on mucin-like glycoproteins, including TAG-72. MAbs CC49 exhibit high reactivity to gastric, pancreatic and colon adenocarcinomas. Due to the high specificity and strong immunoreactivity to target antigen, CC49 antibody has entered in clinical trials for the imaging and treatment of various carcinomas [4]. These data suggest that anti-TAG72 antibodies can be exploited in rational design of the targeted nanogels to improve the selectivity of colorectal cancer imaging.

BODY OF REPORT

This section describes the efforts devoted by the program team to meet the major technical objectives that were: 1) develop the procedures for conjugation of antibodies to nanogels; 2) conjugate antibodies to a nanogel surface; 3) demonstrate specificity of the antibody-nanogels conjugates to immunogen.

Synthesis of nanogels

Diblock copolymer poly(ethylene oxide)-b-poly(methacrylic acid) (PEO$_{170}$-*b*-PMA$_{180}$, M$_w$/M$_n$=1.45, Polymer Source Inc., Canada) was used to prepare polyion complex micelles, templates for the synthesis of nanogels. Nanogels were synthesized using the general procedure as was described earlier [5]. Briefly, PEO-*b*-PMA/Ca^{2+} complexes were prepared by mixing an aqueous solution of PEO170-b-PMA180 with a solution of CaCl$_2$ at a molar ratio of [Ca^{2+}] /[COO−]=1.1. The inner core of the micelles formed was cross-linked through the carboxylic groups using bifunctional agent, 1,2-ethylenediamine, in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) at appropriate molar ratios to achieve the desired theoretical extent of cross-linking. The theoretical extent of cross-linking was 20% and has been controlled by the ratio of amine functional groups to carboxylic acid groups of block-polymer. It should be noted, that actual extent of cross-linking were expected to be significantly less [5], due to side reaction between water and the activated carboxylic groups. Moreover, it is quite likely that some portion of the diamine could form a "loop" being bound to the same PMA chain as well as attach by one amino group giving the free amine. It has been estimated that approximately two cross-links per block copolymer chain were formed in the micelles with targeted 20% degree of cross-linking. The reaction mixture was allowed to stir overnight at room temperature. The byproducts of the cross-linking reaction and metal ions were removed by exhaustive dialysis of the reaction mixtures, against (1) 0.5% aqueous ammonia in the presence of ethylenediaminetetraacetic acid (EDTA), and (2) distilled water. The resulting nanogels were of ca. 160 nm in diameter and had a net negative charge (ζ-potential = -25 mV). The core of such micelles comprised a swollen network of the cross-linked PMA chains and was surrounded by the shell of hydrophilic PEO chains. The particles were stable and revealed no size change even upon a 100-fold dilution.

Procedures for conjugation of antibodies to nanogels

One of the common strategies for immobilization of monoclonal antibodies on the surface of nanoparticles is the attachment through the PEG-linker. In our study MAbs were attached to the surface of nanogels via a bifunctional maleimide-PEG-NH$_2$ (MAL-PEG-NH$_2$) cross-linker which reacts with carboxylic groups of nanoparticles as well as a thiol group

introduced into the antibody. To achieve this, the amino groups from lysine residues of antibody were, first, converted to thiol groups using 2-iminotiolane (Traut's reagent). Briefly, to introduce sulfhydryl groups into antibody, 10-fold molar excess of Traut's reagent was added to solution of antibodies in borate buffer (0.1M, pH 8 with 2-5mM EDTA) and incubated for 1 h at room temperature. Afterwards the thiolated antibodies were purified by Zeba spin desalting columns that has been equilibrated with buffer containing 2-5 mM EDTA. At the next step, the thiolated antibodies were conjugated to PEG linker (M_w 7.5 kDa). During PEGylation of antibodies EDTA was added to all reaction mixtures to prevent oxidation of sulfhydryl groups (i.e., formation of disulfide bonds). The sulfhydryl-reactive antibodies were incubated with 20-fold molar excess of NH_2-PEG-MAL for overnight at 4°C. The resulting PEGylated antibodies were purified by centrifugation (MWCO 30,000 Da, Microcon YM-30) and redispersion in 10 mM phosphate buffer.

Conjugation of antibodies to a nanogel surface

Two different MAbs were used in this study: specific targeting ligand to TAG-72, monoclonal antibody CC49, was coupled to the surface of the nanogels via flexible PEO linker (CC49-clPEO-b-PMA) and nonspecific murine immunoglobulin G (IgG-*cl*PEO-*b*-PMA). The PEGylated MAbs were conjugated with nanogels via amide linkage between the NHS activated carboxyl groups of micelles and amino groups of MAb-PEG (*Figure 1*).
The purified MAb-conjugated nanogels were obtained after size exclusion chromatography (SEC) on Sepharose CL-6B column. It should be noted that the purification of the reaction mixture allowed to remove all unbound MAb, while unmodified and MAb conjugated nanogels are not separated. The size (hydrodynamic diameter) and ζ-potential of the MAb conjugated nanogels were determined by dynamic light scattering (DLS) and data are presented in *Table 1*.
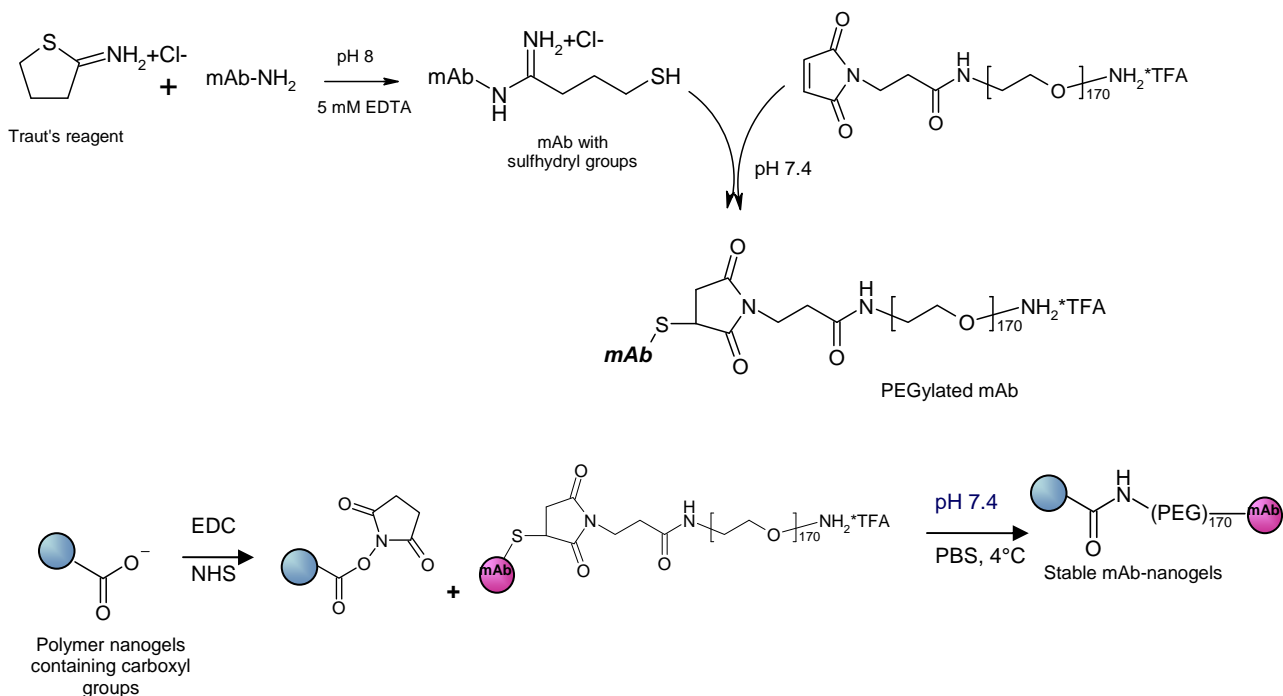
Figure 1. Scheme of synthesis of mAb conjugated nanogels.

Table 1. Physicochemical characteristics of the nanogels at pH 7

Sample	D_{eff} [a],nm	PDI	ζ, mV
*cl*PEO-*b*-PMA	160 ± 3	0.06-0.1	-25.3 ± 3.2
IgG-*cl*PEO-*b*-PMA	125 ± 5	0.08-0.12	-7.8 ± 2.9
CC49-*cl*PEO-*b*-PMA	111 ± 4	0.09-0.11	-8.5 ± 2.5

[a] Effective diameter and ζ-potential values were calculated from five measurements performed on different samples.

After antibody coupling the size of the nanogels was slightly decreased from 160 nm to ca. 110-120 nm. All synthesized nanogels were characterized by relatively low polydispersity indices. The physicochemical characteristics of the both conjugates (IgG-*cl*PEO-*b*-PMA and CC49-*cl*PEO-*b*-PMA) were practically the same. Interestingly, that the negative ζ-potentials of MAb conjugated nanogels were lower than ones of the unmodified nanogels. It is likely that the net negative charge of the MAb modified nanogels was partially shielded by additional external PEG chains and MAb.

A combination of methods has been used for characterization of the targeted polymer micelles. The size (hydrodynamic diameter) and ζ-potential of nanoparticles were determined were performed using a "ZetaPlus" analyzer. To characterize the stability of the micelles in the dispersion, the size measurements have been repeated daily for 10 days. The swelling behaviors of non- and targeted nanoparticles were examined by DLS. The morphology of cross-linked nanoparticles was determined by tapping mode Atomic Force Microscopy (AFM).

The hydrogel-like behavior of the nanogels is observed upon a change of pH [5,6]. Therefore, we further explored the effects of surface modification on the swelling properties of the MAb modified *cl*PEO-*b*-PMA nanogels. The effective diameters and ζ-potential of such nanogels with 20% targeted degree of cross-linking are shown in *Figure 2*.
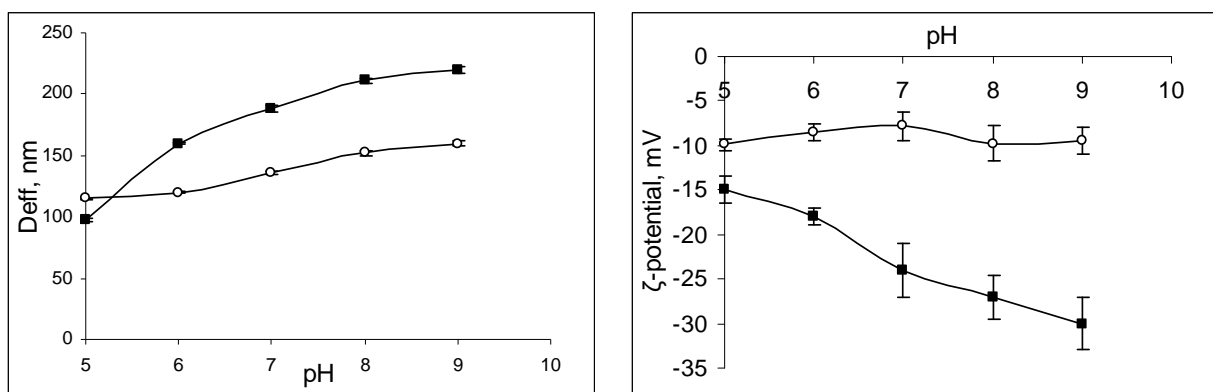

Figure 2. Physicochemical characteristics of *cl*PEO-*b*-PMA nanogels (■) and IgG-*cl*PEO-*b*-PMA (○): effective diameter (D_{eff}) and ζ-potential of nanogels with 20% targeted degree of cross-linking as a function of pH.

The size and net negative charge of non-modified nanogels increased with increasing of pH: the nanogels shown an increase in diameters from 150 nm up to 220 nm when pH of the solution was increased from 6 to 9. Notably, in contrast to the unmodified nanogels, the MAb conjugated nanogels did not reveal pH-dependent swelling behavior: the size and ζ-potential practically did not change in the range of pH values studied. It might be a result of insertion of additional cross-links in the shell or/and core of the nanogels upon the modification. During the cross-linking reaction, the diamine (ED) could attach to the PMA chains by only one amino group giving the free amine. It is possible that activated carboxylic

groups in the nanogels could react with terminal amino group of MAb-PEG (main reaction) as well as with the amino groups of the nanogels (side reaction).

The amount of MAb bounded to nanogels was evaluated by micro BCA assay and was ca. 150-200 μg of MAbs per mg of polymer. The yield of conjugated reaction was calculated based on BCA study and varied from 20 to 50%.
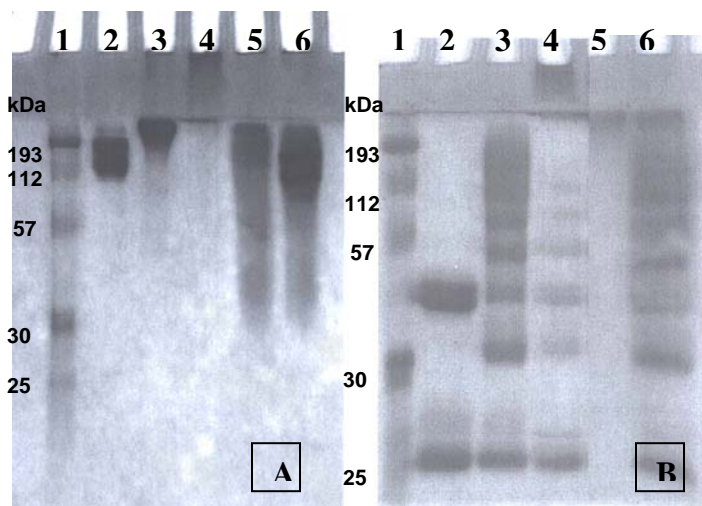

Figure 3. The 4-10% SDS–PAGE under non-denaturing (A) and denaturing conditions (B), followed by Coomassie blue staining. Lanes: protein ladder (1); free IgG (2); IgG-PEG (3); IgG conjugated nanogels (4); free nanogels (5) and mixture of free IgG and nanogels (6).

Each sample has been further analyzed by SDS-PAGE gel electrophoresis without and with reduction by β-mercaptoethanol. Typical electrophoretograms are presented in *Figure 3* (A and B). Free antibody (Immunoglobulin G (IgG) from murine serum, tech. grade > 80%) and unmodified nanogels as well their mixture were used as controls. The free IgG and IgG-PEG shown in lanes 2 and 3 confirmed a successful PEGylation of antibody. As shown in Figure 3B several additional bands appeared upon reducing conditions in the case of PEGylated antibodies, which denote the PEG-modification of light as well as heavy chains of IgG. The IgG conjugated nanogels shown in Figure 3A in lanes 4 and 5 did not migrate due to the high molecular weight of MAb-nanogels constructs. However, no unbound IgG or IgG-PEG was observed in these samples, which indicated a thorough purification by SEC. In contrast, no IgG band retardation was observed in the lanes corresponding to free IgG or their mixture with nanogels. Indeed, under reduction of IgG-nanogels by β-mercaptoethanol the several bands appeared, which resembled light or heavy chains of IgG. These data indicated that the conjugation of the nanogels with monoclonal antibodies (IgG or CC49) using PEG-linker and the purification of MAb-conjugated nanogels were successful.

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To further prove the effective conjugation of antibodies to the nanogels, the conjugation procedure was performed using fluorescently labeled antibodies (FITC-IgG). The FITC-IgG conjugated nanogels were prepared by a similar method and the fluorescence of purified particles was measured using Lambda 25 UV/VIS spectrophotometer. Unmodified nanogels were chosen as a control in this study. The fluorescence spectra of the nanogels are presented in *Figure 4*. As is seen, the fluorescence was detected only for the nanogels modified with FITC-labeled antibodies.

The topology of the MAb modified nanogels was examined by tapping-mode AFM imaging. The purified nanogels (ca. 1mg/ml) were deposited onto positively charged APS-mica and then dried. The typical image of IgG- modified *cl*PEO-*b*-PMA nanogels is presented in *Figure 5*. As expected, the MAbs nanogels retained the spherical morphology and formed flat circular structures upon adsorption on mica surface.
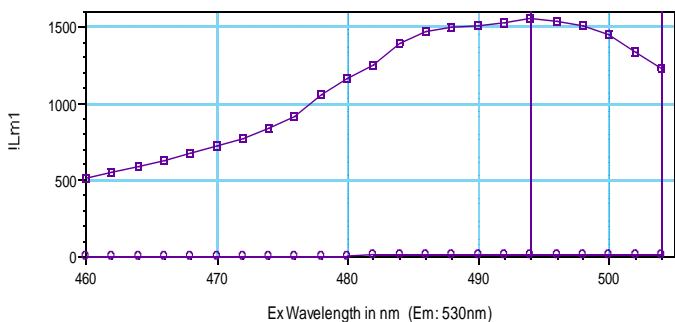

Figure 4. Spectrum of FITC-IgG-micelles (□) and unmodified micelles (○)
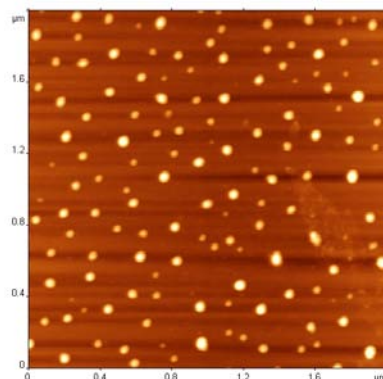

Figure 5. Tapping-mode AFM image of IgG conjugated *cl*PEO-*b*-PMA nanogels. Scan size in 2 μm.

The MAb modified nanogels remained stable in phosphate buffered saline (PBS, pH 7.4) in a wide range of concentrations (up to 1.5 %), exhibiting no aggregation for at least ten days.

Evaluation of antigen-binding selectivity of antibody conjugated nanogels

Specific interaction between antibody modified nanogels and its targeted antigen (bovine submaxillary mucin or BSM) were determined by surface plasmon resonance measurements (SPR). To define the specific binding of the antibody-nanogel conjugates to

BSM as a positive control and bovine serum albumin (BSA) as a negative control[7], the nanogels and their derivatives (IgG or CC49) were prepared. For the experiments, BSM and BSA were immobilized onto a dextran-coated gold sensor chip CM5, which could be regenerated by 100 mM CAPS with no loss of activity. The typical sensograms are presented in *Figure 6*. The analysis of the sensograms revealed that the antibody-conjugated nanogels (CC49-*cl*PEO-*b*-PMA) were able to effectively recognize the immobilized antigen, BSM, as seen by the specific binding of the targeted nanogels with its antigen coated on the sensor chip (Figure 6). However, nanogels lacking the targeting ligand or the nanogels modified with unspecific antibodies (IgG) did not show binding to the BSM antigen.
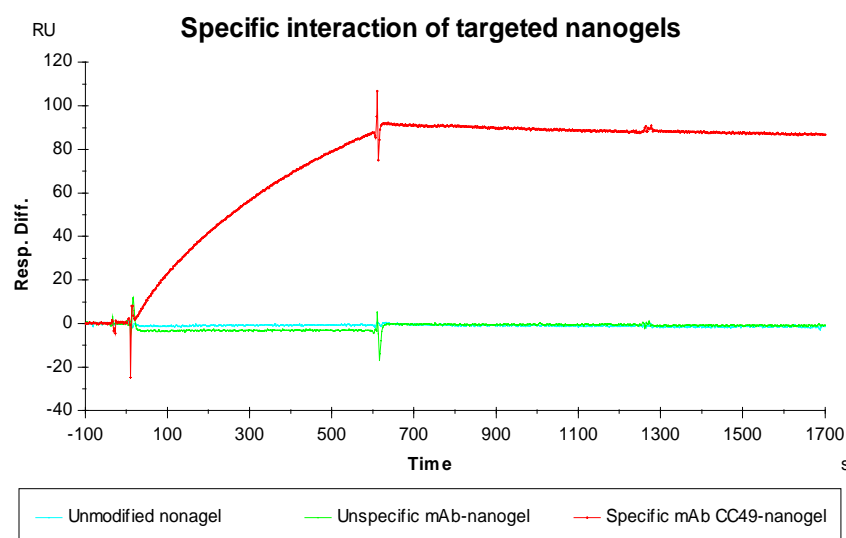

Specific interaction of targeted nanogels

Unmodified nonagel — Unspecific mAb-nanogel — Specific mAb CC49-nanogel

Figure 6. Evaluation of BSM-binding selectivity of MAb conjugated nanogels by SPR measurements. Eluent: HBS-EP buffer, pH 7.4; flow rate: 5 µl min^{-1}; density of BSA & BSM was approx. 0.6 ng mm^{-2} per channel; sample concentration was 60 ug/ml.

Interestingly, CC49-*cl*PEO-*b*-PMA once attached to the BSM remained on the chip surface even though excess amount of free MAb CC49 was added. On the other part, when both CC49-*cl*PEO-*b*-PMA and free CC49 (in the concentration range of 100-500 nM) were injected as a mixture, the binding effect of CC49 conjugated nanogels was partially decreased (*Figure 7*). These results suggest that specific CC49 modified nanogels can bind strongly to BSM and could not be displaced or completely blocked by free CC49. Such behavior was not observed in the case of IgG-*cl*PEO-*b*-PMA (negative control) or unmodified nanogels. It should be noted that all sensorgrams were described as a specific binding of samples to BSM subtractive to non-specific binding to BSA. The dissociation

constants (K_D) for MAb CC49 and CC49-*cl*PEO-*b*-PMA nanogels were 8 nM and 10.3 nM, respectively, as determined by real-time kinetic analysis using BIAcore software.
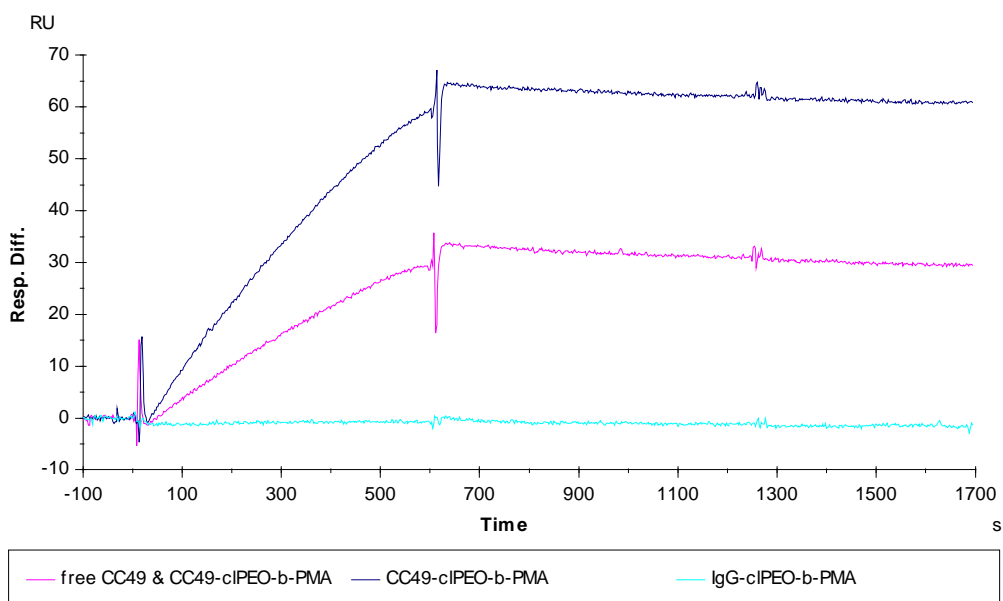

Figure 7. Blocking study of the binding of the modified nanogels to the BSM sensor chip. Eluent: HBS-EP buffer, pH 7.4; flow rate: 5 µl min^{-1}; density of BSA & BSM - 0.6 ng mm^{-2} per channel; sample concentration - 60 ug/ml; concentration of free CC49 was 250 nM.

Taken together, the SPR analysis of antibody conjugated nanogels clearly revealed that CC49-*cl*PEO-*b*-PMA nanogels exhibit selective specific binding to its antigen; and this approach can allow specific recognition of TAG-72.

KEY RESEARCH ACCOMPLISHMENTS

- Procedures for the conjugation of the nanogels with MAbs in aqueous media were developed.
- The CC49 antibodies to TAG72 specific to colon cancer cells were successfully attached to the nanogels through the PEG-linker.
- The purification methods were developed. It was demonstrated that the developed synthetic approach for the MAb conjugation to the nanogels provides reproducible results.

- The physicochemical characteristics of the MAb CC49-modified nanogels (dimensions, swelling behavior) were determined by combination of different physico-chemical techniques (DLS, AFM, fluorescence spectroscopy).

- Specific binding of the MAB CC49-nanogel conjugates to bovine submaxillary mucin (BSM), which contains the epitopes recognized by CC49 antibodies, as a positive control and bovine serum albumin (BSA) as a negative control, was determined by surface plasmon resonance measurements. The binding ability of the nanogels conjugated with CC49 was compared to that of unconjugated constructs.

- The dissociation constants (K_D) for free MAb CC49 and CC49-conjugated nanogels were 8 nM and 10.3 nM, respectively,

REPORTABLE OUTCOMES

- The procedures for the conjugation of the antibodies to the nanogels were developed.
- Anti-TAG72 antibodies (MAb CC49) were successfully conjugated to the nanogels.
- MAb CC49-conjugated and nonspecific IgG-conjugated nanogels were synthesized, purified, and characterized.
- Specific recognition of antigen by CC49-modified nanogels was demonstrated.

CONCUSIONS

The robust procedures for the incorporation of targeting ligands, monoclonal antibodies, into the nanogels using flexible hydrophilic PEO linkers were developed. Heterobifunctional PEO with maleimide- and amine-modified terminal groups was used to react with sulfhydryl group of the anibidies to form stable thioether bonds. The amino-functionalized PEO-antibody constructs were further covalently attached to the acid residues within the core of nanogels using EDC –mediated coupling reaction. The physicochemical characteristics of nanogels (dimensions, surface charge, swelling behavior) were evaluated and extend of antibody modification was calculated. SDS-PAGE electrophoresis revealed successful modification and purification of such systems. The SPR analysis confirmed a specific binding of the CC49 antibody conjugated nanogels to its antigen, bovine submaxillary mucin.

METHODS AND MATERIALS

Diblock copolymer poly(ethylene oxide)-b-poly(methacrylic acid) (PEO170-b-PMA180) (Mw/Mn=1.45) was purchased from Polymer Source Inc., Canada. The block lengths were 170 and 180 repeating units for PEO and PMA respectively. The concentration of carboxylate groups in the copolymer samples was estimated by potentiometric titration. Sodium cyanoborohydride, 2-Iminothiolane hydrochloride (Traut's reagent), micro BCA (bicinchoninic acid) protein colorimetric assay kit, Zeba spin desalting columns were purchased from Thermo Fisher Scientific (Rockford, IL). Maleimide-PEG$_{7500}$-NH$_2$, TFA salt (MAL-PEG-NH$_2$) was prepared by Creative PEGWorks (Winston Salem, NC). Immunoglobulin G (IgG) from murine serum, Sepharose CL-6B, disposable NAP-10 desalting columns, 1,2-ethylenediamine, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), bovine submaxillary mucin (BSM), and other chemicals were purchased from Sigma–Aldrich (St Louis, MO) and were used as received. The CC49 IgGs were developed in Dr. Batra's group by the immunization of mice with purified TAG-72 (see previous report).

Purification and determination of antibody extent

After conjugation of cross-linked micelles with antibodies, the size exclusion chromatography was used to separate antibody conjugated polymer micelles from free antibodies. For this, Sepharose CL-6B column was equilibrated with PBS (pH 7.4) and run at 1ml/min. Initially the column has been calibrated with Dextrose Blue (Mw 2,000 kDa) and alcohol dehydrogenase yeast (Mw 150 kDa) which correspond to molecular weight of cross-linked polymer micelles and antibody, correspondingly. The samples were monitored by UV (260 nm), appropriate fractions were collected and concentrated by centrifugal filtration using centrifugal filter devices (MWCO 30,000 Da, Millipore). The amount of antibody in each fraction was determined by colorimetric micro BCA (bicinchoninic acid) protein colorimetric assay. The purified samples and standard (BSA) were diluted in 10 mM phosphate buffer. The samples were incubated with dye at 37°C for 2 h and absorbance was measured at 562 nm in SPECTRA MAX M5 reader. The fractions of targeted nanoparticles were freeze dried and a concentration of ug antibody per mg polymer was calculated.

SDS-PAGE

SDS-PAGE gel electrophoresis was performed under reducing (5% 2-mercaptoethanol) and non-reducing conditions. The gels were run according to the method of Laemmli [8] using 10 % polyacrylamide gel with a stacking gel of 4% acrylamide. Protein bands have been visualized by Coomassie Blue stain.

Electrophoretic mobility and size measurements

Electrophoretic mobility measurements were performed using a "ZetaPlus" analyzer (Brookhaven Instrument Co.) with a 30 mW solid-state laser operating at a wavelength of 635 nm. Zeta-potential (ζ) of the nanogels was calculated from the electrophoretic mobility values using Smoluchowski equation. Effective hydrodynamic diameters (D_{eff}) of the nanogels were measured by photon correlation spectroscopy (DLS) in a thermostatic cell at a scattering angle of 90° using the same instrument equipped with a Multi Angle Sizing Option (BIMAS). All measurements were performed at 25 °C. Software provided by the manufacturer was used to calculate the size of the particles and polydispersity indices. The diameters mean values were calculated from the measurements performed at least in triplicate.

Stability of MAb conjugated nanogels

The stability of MAb conjugated nanogels was assessed in vitro. The dispersions of both MAb-conjugated nanogels and unmodified nanogels were prepared and incubated at 37°C in PBS for 10 days. The particle size was analyzed daily by DLS.

Atomic force microscopy (AFM)

The morphology of nanogels was determined by AFM. Samples for AFM imaging were prepared by depositing 5 μl of an aqueous dispersion of micelles (ca.1 mg/ml) onto positively charged 1-(3-aminopropyl)silatrane mica surface (APS-mica) for 2 min followed by surface drying under argon atmosphere. The AFM imaging was performed in air and liquid using a Multimode NanoScope IV system (Veeco, Santa Barbara, CA) operated in a tapping mode. The imaging in air was performed with regular etched silicon probes (TESP)

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with a spring constant of 42 N/m. The images were processed and the widths and heights of the particles were measured using Femtoscan software (Advanced Technologies Center, Moscow, Russia).

Surface Plasmon Resonance Measurements (SPR)

    To demonstrate the specific binding of the antibody-micelles conjugates to bovine submaxillary mucin (BSM), which contains the epitopes recognized by MAb CC49 [9], as a positive control and bovine serum albumin (BSA) as a negative control, the surface plasmon resonance measurements have been performed [7].

Immobilization of surface. BSA and BSM were immobilized on the surface of a dextran-coated gold film (sensor chip CM5) according the general procedure for immobilization by amine coupling provided by manufacturer. Briefly, after equilibration of instrument with HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4) following samples were injected into the BIAcore: (i) 35 ul NHS/EDC in a mixture solution (1:1) to activate the carboxylated dextran; (ii) 30 ul BSM or BSA dissolved at a concentration 70 or 12.5 ug/ml in 10 mM sodium acetate, pH 4.5 and pH 4, respectively; (iii) 35 ul of 1M ethanolamine hydrochloride in water (pH 8.5) to deactivate residual NHS-ester on sensor chip and desalting of electrostaticlly bound proteins. The immobilization was performed at a flow rate of 5 ul/min at 25°C, allowing the binding of 600 RUs of proteins per channel.

Binding analysis. The binding was performed in the concentration range of 0-500 nM of mAb conjugated micelles (on the basis of antibody concentration) at a flow rate of 5 ul/min,run in triplicate for each sample. The particles were allowed to interact with the BSM or BSA for 10 min and dissociated time was 5 min. The sensor surface was regenerated with 25-100 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid) at a flow rate of 5 ul/min with no loss of activity. To study the interaction of polymer micelles, the non-specific IgG conjugated and unmodified polymer micelles were prepared as controls. To study the specificity of the interaction, binding experiments were performed in the presence of 200 nM of free CC49. The kinetic constants for association (K_A) and dissociation (K_D) were evaluated using the BIAevaluation 3.0.2 software package (BIAcore, Piscataway, NJ, USA) where the external design correlated with the Langmuir 1:1 binding model [10].

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